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Salivaricin D, a Novel Intrinsically Trypsin-Resistant Lantibiotic from *Streptococcus salivarius* 5M6c Isolated from a Healthy Infant

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In this work, we purified and characterized a newly identified lantibiotic (salivaricin D) from *Streptococcus salivarius* 5M6c. Salivaricin D is a 34-amino-acid-residue peptide (3,467.55 Da); the locus of the gene encoding this peptide is a 16.5-kb DNA segment which contains genes encoding the precursor of two lantibiotics, two modification enzymes (dehydratase and cyclase), an ABC transporter, a serine-like protease, immunity proteins (lipoprotein and ABC transporters), a response regulator, and a sensor histidine kinase. The immunity gene (*salI*) was heterologously expressed in a sensitive indicator and provided significant protection against salivaricin D, confirming its immunity function. Salivaricin D is a naturally trypsin-resistant lantibiotic that is similar to nisin-like lantibiotics. It is a relatively broad-spectrum bacteriocin that inhibits members of many genera of Gram-positive bacteria, including the important human pathogens *Streptococcus pyogenes* and *Streptococcus pneumoniae*. Thus, *Streptococcus salivarius* 5M6c may be a potential biological agent for the control of oronasopharynx-colonizing streptococcal pathogens or may be used as a probiotic bacterium.

Streptococcus salivarius is a member of the lactic acid bacteria (LAB) that forms part of the normal flora of the oral cavity, throat, and upper respiratory tract (23, 28, 40, 44). It has also been observed in the nasopharynx and intestinal tract and isolated from the human feces (25, 35, 44) and breast milk of healthy women (1, 46). *S. salivarius* strains produce a number of bacteriocins, most of which are lantibiotics (22, 39, 52–54).

Lantibiotics are small, heat-stable, ribosomally synthesized, posttranslationally modified antimicrobial peptides (bacteriocins) produced by Gram-positive bacteria (3). The lantibiotics, unlike other bacteriocins, are characterized by containing the thioether amino acids lanthionine (Lan) and 3-methyllanthionine (MeLan) and the modified amino acids didehydroalanine (Dha) and didehydrobutyrine (Dhb) (55). Lantibiotics are initially synthesized as inactive linear prepeptides that undergo subsequent extensive modifications to be biologically active. The modifications involve mainly dehydration of serine and threonine residues, forming the didehydro amino acids Dha and Dhb, respectively, which react with the nearby C-terminally located cysteine residues (seen among linear lantibiotics) to form a thioether linkage, which results in the formation of Lan and MeLan, respectively. Finally, the modified peptide is exported and cleaved from its leader in order to be active.

According to a very recent classification system, lantibiotics and lantipeptides (class Ia), consist of four subclasses (38). Subclass I lantibiotics are modified by two different enzymes, LanB enzyme (dehydratase) and LanC enzyme (cyclase), exported by LanT, and their leader peptides are removed by the LanP enzyme. Subclass II lantibiotics are modified by a single enzyme (LanM) which has both dehydratase and cyclase activity and is exported by LanT, which also cleaves the leader peptides. Subclasses III and IV consist of lantibiotic-like peptides such as morphogenetic peptides and lantipeptides, both of which lack antimicrobial activity.

A biosynthetic gene cluster that consists of genes encoding the prepeptide (LanA), one (LanM) or two (LanB and LanC) modification enzymes that introduce the thioethers, an ABC transporter (LanT) which exports the bacteriocin and removes the leader or is dedicated for export function only, an extracellular protease (LanP) that removes the leader, and finally a self-protecting system of the producer referred to as immunity proteins [LanI(H) and/or LanFE(G)] is necessary for lantibiotic production (3). Many lantibiotics are regulated by a quorum-sensing system consisting of a response regulator (LanR), a histidine protein kinase (LanK), and a peptide pheromone (induction peptide) that sometimes can be the bacteriocin itself (32).

Lantibiotics kill mainly Gram-positive bacteria. Some (e.g., nisin and planosporicin) are also active against Gram-negative bacteria (7). In general, lantibiotics kill target cells by inhibiting cell wall biosynthesis by binding to the cell wall precursor lipid II and/or by formation of pores in cell membranes, leading to efflux of small molecules and dissipation of membrane potential (2, 3, 5).

Bacteriocins may have potential applications in the food industry to control food spoilage and food-borne infections (8) and in medicine as selective antimicrobials to inhibit pathogens with no or little effect on the normal flora, unlike classical antibiotics that have broad-spectrum activity (37). Bacteriocin production is considered a probiotic feature, and it has convincingly been shown that bacteriocin-producing probiotic lactic acid bacteria can inhibit growth of *Listeria monocytogenes* in mice (10). Thus, identification and characterization of bacteriocin-producing LAB of human origin are needed not only to develop probiotic bacteria with diverse antimicrobial potentials but also to develop bacteriocins into chemotherapeutic agents to control infections (12, 47). To this end, we aimed at isolation of bacteriocin-producing LAB from healthy human infants. In this study, a new bacteriocin (lan-

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TABLE 1 Inhibition spectrum of salivaricin D

Indicator	Inhibition[a]	MIC (nM)[b]
Bacillus subtilis BD630	+	1.3
Bacillus subtilis OG1	+	5.1
Clostridium bifermentans NCDO 1711	+	0.01
Clostridium butyricum NCDO 855A	+	6.4
Enterococcus faecalis V583	−	
Lactobacillus curvatus 89	+	0.23
Lactobacillus curvatus NCDO 2739	+	7.2
Lactobacillus curvatus subsp. *curvatus* BCS35	+	0.45
Lactobacillus sake NCDO 2714	+	0.14
Lactococcus lactis NCDO 1403	−	
Lactococcus lactis NCDO 497	−	
Lactococcus lactis subsp. *cremoris* 393	+	72.1
Lactococcus lactis subsp. *cremoris* 730	+	18.1
Leuconostoc lactis NCDO 533	+	0.1
Leuconostoc NCDO 543	+	1.8
Listeria innocua BL86/26 B	−	
Micrococcus luteus ATCC 4698	+	ND
Staphylococcus aureus 2002-60-8452	−	
Streptococcus mitis NCTC 12261	−	
Streptococcus oralis SK 153	−	
Streptococcus pneumoniae D39	+	0.03
Streptococcus pneumoniae TIGR4	+	0.06
Streptococcus pneumoniae R6	+	12
Streptococcus pyogenes 08198	+	7.2
Streptococcus pyogenes NCTC 700294	+	ND
Streptococcus sanguis SK 36	−	
Streptococcus suis	+	0.11
Streptococcus thermophilus	+	ND
Streptococcus thermophilus	+	ND

[a] +, inhibition; −, no inhibition.
[b] ND = not determined.

tibiotic) was purified from *S. salivarius* 5M6c and characterized at the biochemical and molecular levels.

MATERIALS AND METHODS

Isolation and identification of bacteriocin-producing strains. Bacteriocin-producing strains were isolated from a fecal sample obtained from a healthy Ethiopian infant. A 10-fold serial dilution of the fecal sample was done in MRS broth, after which 0.1 ml from 10^{-5}, 10^{-6}, and 10^{-7} dilutions each was plated on MRS agar and anaerobically incubated at 37°C. Three colonies with different morphologies were randomly picked from each plate, checked for purity, and tested for bacteriocin production. Species identification was done by using partial 16S rRNA gene sequencing and BLAST analysis.

Bacterial strains and culture conditions. Salivaricin D-producing *S. salivarius* isolates tested were 5M6c, 5M6a, 5M5a, 5M5c, and 5M7a. Bacterial strains tested for salivaricin D sensitivity are given in Table 1. Solid media and soft agar were prepared with 1.5% and 0.7% agar, respectively. All strains were maintained as frozen stocks at −80°C in 13% glycerol. *Enterococcus* and *Listeria* species were grown in GM17 (M17 supplemented with 0.5% glucose) at 37°C. *Clostridium*, *Lactobacillus*, *Pediococcus*, and *Leuconostoc* species were grown in MRS broth at 30°C. *Lactococcus* species were grown in GM17 at 30°C. *Streptococcus* species were grown in MRS, GM17, brain heart infusion (BHI), or Todd-Hewitt (TH) broth at 37°C. *Staphylococcus* and *Bacillus* species were grown in BHI broth at 30°C. *Escherichia coli* was grown in LB medium. All media were purchased from Oxoid (England).

Bacteriocin/antimicrobial activity assay. Antimicrobial activity was detected using a soft agar overlay assay. The isolated bacteria were spotted onto MRS agar plates and incubated for 16 h at 37°C. The resulting colonies were overlaid with MRS soft agar containing an overnight culture (~2×10^7 cells/ml) of the indicator strain *Lactobacillus sakei* NCDO 2714. After overnight incubation, the colonies were examined for the formation of growth inhibition zones, as an indication of antimicrobial activity. To investigate whether the antimicrobial activity observed was caused by a nonprotein compound or a bacteriocin, the antimicrobial activity was tested in the presence of proteinase K (20 mg/ml).

Bacteriocin activity was quantified by microtiter assay as described previously (20), using *L. sakei* NCDO 2714 as an indicator strain. One bacteriocin unit (BU) was defined as the amount of salivaricin D (0.14 nM) (Table 1) that inhibited the growth of the NCDO 2714 indicator strain by 50%. Similarly, the MIC of a purified bacteriocin against sensitive strains was calculated using the microtiter assay. MIC was defined as the concentration of a pure bacteriocin that inhibited the growth of a sensitive strain by 50%.

Bacteriocin purification. A 2-liter overnight culture (22 h) of the bacteriocin producer, *Streptococcus salivarius* 5M6c, was grown in MRS broth, and supernatant was collected by centrifugation at 9,500 × *g* at room temperature for 10 min and filtered with a 0.22-μm filter (Millipore). The total protein was precipitated by ammonium sulfate as describe previously (4) and collected by centrifugation at 29,800 × *g* at 4°C for 30 min. The resulting pellet was resuspended in 200 ml sterile distilled water, and the pH was adjusted to 3.5 with 1 M HCl.

Bacteriocin purification was done by ion-exchange chromatography and reversed-phase chromatography (RPC) as described previously (4), with the following modifications. The sample was passed through a 10-ml SP Sepharose Fast Flow system and washed with 10 mM sodium phosphate buffer at pH 6. Reversed-phase chromatography was done in the following 3 steps: RPC I to RPC III. The columns used for RPC I and RPCs II and III were 3-ml Resource 15 RPC and 5-μm Sephasil Peptide C_8 ST 4.6/250 columns (both from Pharmacia Biotechnology), respectively. Fractions (0.5 ml) from RPCs II and III were eluted with 7-column-volume linear gradients with from 25 to 45% and from 25 to 35% isopropanol, respectively.

Mass spectrometry, N-terminal sequencing, and amino acid composition analysis. The molecular weights of the purified bacteriocins (fractions 2, 3, 6, 7, and 8) (Fig. 1) were determined by mass spectrometry as described previously (13). Peptide mass similarity searches were conducted at ExPASy using the TagIdent tool (http://web.expasy.org/tagident/). In order to determine the presence and estimate the number of dehydrated amino acids, salivaricin D was derivatized with ethanethiol, and the resulting change in mass was analyzed by matrix-assisted laser

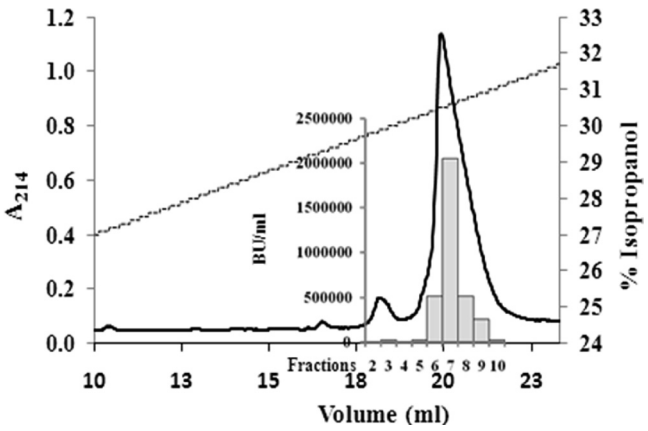

FIG 1 The chromatographic elution profile from RPC III shows two bacteriocin peaks. The large absorbance peak with elution at 32.5% is salivaricin D, and the smaller peak to the left that eluted at 30% is a second and unknown bacteriocin-like activity. The solid line and the broken line show the absorbance at 214 nm and the elution gradient of isopropanol (in percentage), respectively. The antimicrobial activity is shown as gray columns.

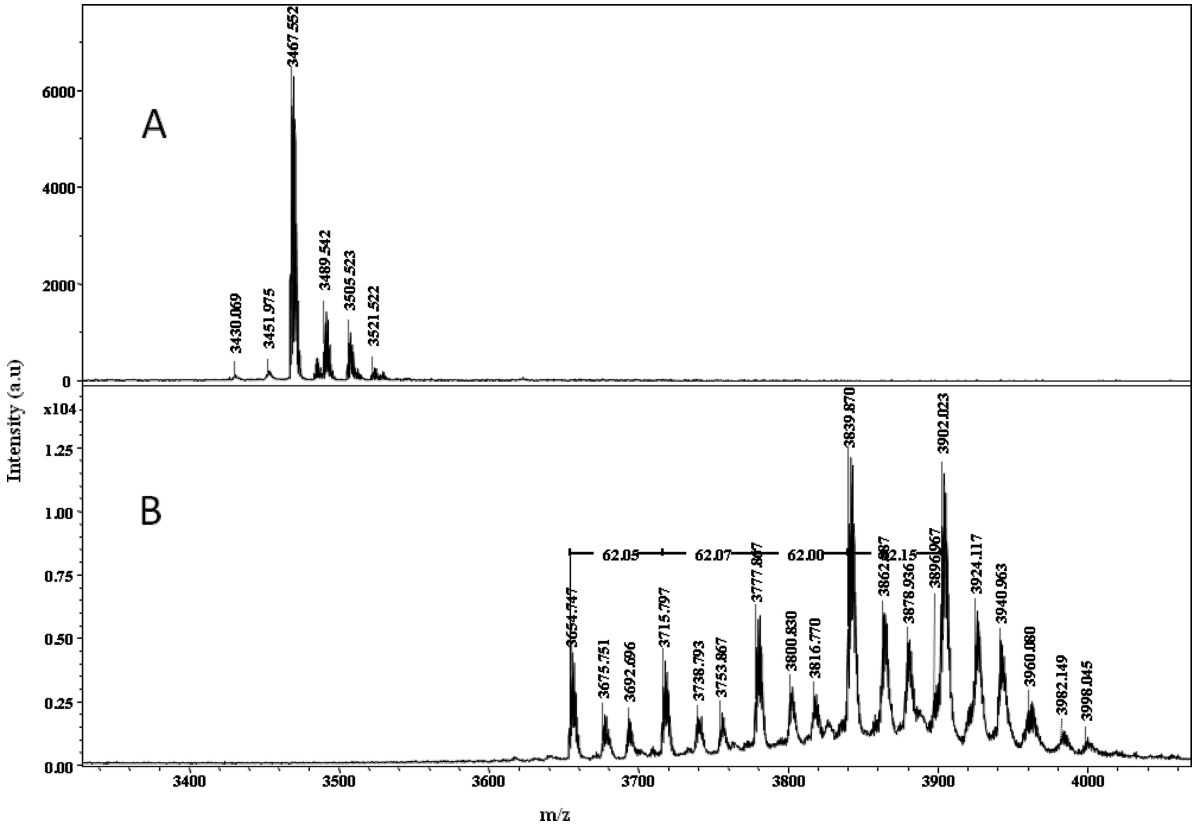

FIG 2 MALDI-TOF mass spectrometry analysis of salivaricin D and its ethanethiol-derivatized products. (A) Native salivaricin D; (B) salivaricin D derivatized with ethanethiol.

desorption ionization–time of flight (MALDI-TOF) mass spectrometry (30). This information was used to determine if salivaricin D was a lantibiotic.

Amino acid composition analysis and N-terminal sequencing (Edman degradation) were performed with purified salivaricin D by Alphalyse A/S, Denmark. Amino acid composition analysis was done as described below. The peptide was hydrolyzed in 6 N HCl, 0.1% phenol, and 0.1% thioglycolic acid at 110°C for 20 h, followed by identification and quantification of amino acids with a BioChrom 30 amino acid analyzer using ion-exchange chromatography, postcolumn derivatization with ninhydrin, and detection at 570 nm and 440 nm. The nonnatural amino acid norleucine (Nle) was used as an internal control standard. N-terminal sequencing was performed with an ABI Procise 494 sequencer using a polyvinylidene fluoride (PVDF) membrane.

Genomic DNA isolation for PCR. Genomic DNA isolation was done, with modification of the protocol used with the bacterial genomic DNA purification kit (Edge Biosystems). Briefly, a 1.5- to 3-ml culture was pelleted, washed in 500 μl TES buffer (10 mM Tris-HCl at pH 8, 1 mM EDTA, 100 mM NaCl), resuspended in 200 μl spheroplast buffer (10% sucrose, 2 mg/ml lysozyme, 0.4 mg/ml RNase A, 25 U/ml mutanolysin, 25 mM Tris at pH 8.0, 25 mM EDTA at pH 8.0), and incubated at 37°C for 10 to 20 min until cell lysis occurred. Then, 50 μl of each of 5% SDS and 5 M NaCl was added, mixed, and incubated at 65°C for 10 min. Buffer N3 (100 μl) (Qiagen) was then added, and samples were mixed and centrifuged in a microcentrifuge at maximum speed at 4°C for 15 min. The supernatant was transferred to a new tube, mixed with an equal volume of isopropanol, and centrifuged in a microcentrifuge at maximum speed at room temperature for 15 min to precipitate the DNA. The resulting pellet was washed with 70% ethanol by centrifugation in a microcentrifuge at maximum speed at room temperature for 10 min. The final pellet was air dried and resuspended in 1× TE buffer, pH 8.

Genome sequencing and draft assembly. Approximately 100 μg of total DNA from *Streptococcus salivarius* 5M6c was purified using a Qiagen genomic tip according to the manufacturer's recommendations, with minor modifications made to optimize lysis as described above.

Genome sequencing was performed (GATC Biotech) using a combination of 454 Life Sciences pyrosequencing and Illumina technologies. The data sets obtained are as follows: using GS FLX Titanium, 474,256 shotgun reads, average read length of 253 nucleotides (nt), and average coverage of 50; an 8-kb mate pair read library with 26,013 reads and an average read length of 128 nt; and an Illumina GA pair end library, 656,3621 reads, average read length of 31 nt, and average coverage of 93. *De novo* assembly (GATC Biotech) was performed using the GS FLX shotgun data, generating 107 large contigs.

Identification of the salivaricin D gene cluster by genome scanning. The contig 00059 of the GS FLX Titanium genome sequencing of *S. salivarius* 5M6c was found to carry the genes needed for the salivaricin D biosynthesis. The identification was performed by BLAST searches for the presence of LanA, LanB, and LanC homologs. Putative salivaricin D gene clusters in contigs of the genome were analyzed by sequence alignment to nisin family lantibiotic biosynthesis clusters like nisin A. Gene prediction was performed with candidate contigs using Prodigal (21). Manual inspection of gene calling was performed using Artemis (41). Gene functions were assigned based on homology searches using Conserved Domain BLAST (29) and BLASTp (see Table 3). The deduced amino acid sequence of candidate salivaricin D structural genes were compared to the observed mass of purified salivaricin D, and the number of dehydrated residues was determined by ethanethiol derivatization of pure salivaricin D (Fig. 2).

Cloning and heterologous expression of the salivaricin D immunity gene. In order to confirm the function of the immunity gene (*slvI*), it was cloned and expressed in the sensitive indicator strain *L. sakei* NCDO 2714.

A 755-bp fragment that includes the *slvI* gene was amplified from genomic DNA of *S. salivarius* 5M6c using the following primers: salxI-f1, 5′-GGT GGTGTCGACTAGAAAGGAATCTAAATGGGACGAC-3′, and salxI-r1, 5′-GGTGGTGCATGCTCATCCTACTCTTCCTTCATTGCAC-3′ (SalI and SphI restriction sites are underlined). The resulting PCR product was digested with the restriction enzymes and ligated into pMG36e (50), which had been linearized by the same enzymes using standard protocols. The resulting recombinant plasmid will be here referred to as pLG500. One Shot TOP10 chemically competent *E. coli* cells (Invitrogen) were transformed with plasmid pLG500. Positive transformants were selected and subcultured in selective LB broth with 200 μg/ml erythromycin. The plasmid pLG500 was extracted from the *E. coli*, and the presence of the insert was checked by restriction digestion with SalI and SphI. The integrity of the insert was confirmed by DNA sequencing. *L. sakei* NCDO 2714 was then transformed with the plasmid pLG500, using pMG36e (without the insert) as a control. Positive clones were selected on MRS broth supplemented with 10 μg/ml erythromycin and subsequently grown in selective MRS broth. The plasmid pLG500 was extracted from *L. sakei* NCDO 2714, and the presence of the insert was checked by PCR. The sensitivity of the *L. sakei* NCDO 2714 clone containing pLG500 or pMG36e to purified salivaricin D was tested using soft agar overlay and microtiter assays (as described above).

Nucleotide sequence accession number. The deposited GenBank accession number for contig 00059 that contains a salivaricin D locus is JN564797.

RESULTS

Bacteriocin production. In an attempt to screen fecal lactic acid bacteria of healthy infants for bacteriocin production and potential probiotics, we identified *S. salivarius* 5M6c (and other *S. salivarius* isolates from one of the infants), which produced a novel bacteriocin that we called salivaricin D. Based on plate counting, a total of 10^7 to 10^9 CFU/g feces was obtained. Colonies were isolated from three MRS agar plates at the highest dilutions, and 5 out of 9 colonies were found to be bacteriocin-producing *S. salivarius* isolates. The result suggests a high prevalence of bacteriocin-producing *S. salivarius* in the fecal sample. The bacteriocin was produced at 30°C or 37°C in GM17, MRS, BHI, and TH broth, although the maximum production was observed in GM17 broth (about 10,000 BU/ml).

Bacteriocin purification. The third step of reversed-phase chromatography (RPC III) revealed the presence of two peaks with antimicrobial activity, suggesting the presence of two bacteriocins-like activities. They eluted at 30% and 32.5% isopropanol (Fig. 1), and mass spectrometry analyses gave masses of 3,467.55 Da (Fig. 2) and 3,483.02 Da (data not shown), respectively. Salivaricin D (eluted at 32.5%) was selected for further analysis because it was produced in significantly larger amounts and had a broader antibacterial activity. The minor activity might be an oxidized form of salivaricin D since its molecular mass is approximately 16 Da larger than that of salivaricin D.

Biochemical properties of salivaricin D. Salivaricin D is sensitive to proteinase K digestion but not to trypsin. It does not absorb UV at 280 nm, indicating the absence of tryptophan and tyrosine residues in the peptide. The activity of the bacteriocin was not destroyed by heating at 70°C for 15 min and remained fully active after storage at 4 to 6°C for at least 10 months or at room temperature (22 to 24°C) for at least 3 weeks.

Salivaricin D inhibition spectrum and MICs. Salivaricin D is a relatively broad-spectrum bacteriocin that showed inhibitory activity against members of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Micrococcus*, *Bacillus*, and *Clostridium* but not against *Staphylococcus* (except some activity against *Staphylococcus carnosus*), *Enterococcus*, and *Listeria* (see Table 3). Interestingly, this bacteriocin has a very low MIC value and effectively kills important pathogens like *S. pyogenes* (MIC, 7.2 nM) and *S. pneumoniae* (MIC, 0.06 to 1 nM). The bacteriocin-like activity that elutes at 30% (Fig. 1, fractions 2 and 3), which has absorbance at 280 nm, inhibited the growth of only two of the tested bacteria (*L. sakei* and *Bacillus coagulans*). Furthermore, the two bacteriocins did not act synergistically (data not shown).

TABLE 2 Amino acid analysis of salivaricin D

Amino acid	Experimental no. of amino acid residues (%)[a]	No. of amino acid residues deduced
Asp/Asn	1 (3.8)	1
Thr[b]	0 (0)	3
Ser[b]	0 (0.2)	4
Glu/Gln[c]	3–4 (13.9)	1
Gly	4 (15.8)	4
Ala	0 (0.2)	0
Cys	ND	4
Val	2 (7.8)	2
Met	1 (3.6)	1
Ile	4 (17.7)	5
Leu	2 (8.1)	2
Tyr	0 (0)	0
Phe	1 (3.5)	1
His	3–4 (14.7)	4
Lys	1 (3.8)	1
Agr	0 (0)	0
Pro	2 (6.8)	1
Trp	ND	0

[a] ND, not determined.
[b] All residues were dehydrated/modified and consequently not accessible for detection.
[c] The elution peak of Glu/Gln was probably contaminated with some of the modified amino acid residues (the same retention time).

N-terminal sequencing and amino acid analysis. Since the mass of salivaricin D is similar to those of many known lantibiotics such as nisin, bovicin HJ50, and pep5, we hypothesized that salivaricin D might be a lantibiotic. However, it was not possible to detect the genes carried by the salivaricin D locus by PCR using primers designed with the above-mentioned lantibiotics. Peptide sequencing by tandem mass spectrometry did not work because of poor fragmentation of salivaricin D and its resistance to trypsinization. Only the N-terminal amino acid was determined to be phenylalanine by Edman degradation, but further sequencing failed probably due to the presence of a modified residue in the peptide. Amino acid composition analysis indicated the absence of arginine, tyrosine, alanine, serine, and threonine (Table 2). The absence of Ser and Thr provided additional evidence that salivaricin D is a lantibiotic, since these residues usually undergo dehydration in lantibiotics.

Presence of modified amino acid residues in salivaricin D. Derivatization of salivaricin D with ethanethiol (mass = 62.134 Da) revealed a mass difference of 434.47 Da between unmodified ($M+H^+$ = 3,467.55 Da) and completely modified ($M+H^+$ = 3,902.02 Da) peptides (Fig. 2). This indicated the presence of 7 modified amino acid residues (modification sites) in salivaricin D and that salivaricin D is a lantibiotic. Ethanethiol addition to three dehydrated amino acid residues was complete for all molecules of salivaricin D and increased its molecular mass from 3,467.5 to

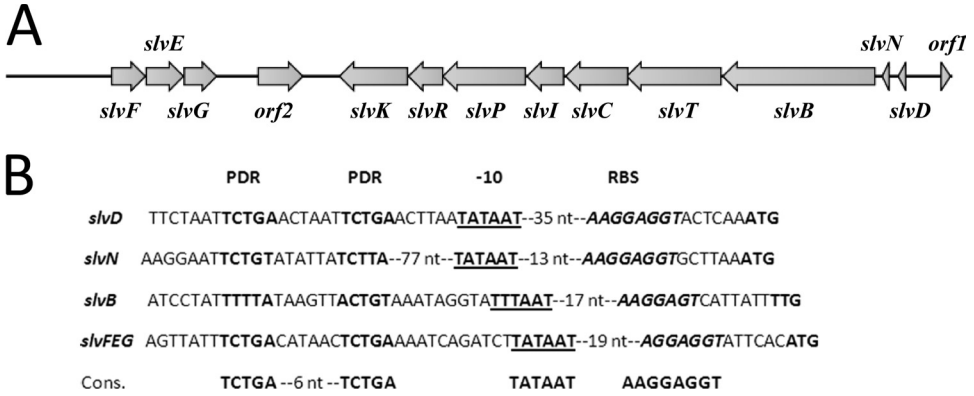

FIG 3 (A) Genetic organization of the salivaricin D locus. The ORFs represent genes that encode the following: salivaricin D precursor (*slvD*), 174 bp; salivaricin N precursor (*slvN*), 162 bp; a dehydratase (*slvB*), 2,955 bp; an ABC transporter (*slvT*), 1,839 bp; cyclase (*slvC*), 1,221 bp; immunity protein (*slvI*),735 bp; a protease (*slvP*), 1,617 bp; a response regulator (*slvR*), 687 bp; a sensor histidine kinase (*slvK*), 1,338 bp; and ABC transporter immunity proteins (*slvG*, 651 bp; *slvE*, 744 bp; and *slvF*, 675 bp). (B) Alignment of putative promoter sequences. Putative ribosome binding sites (RBS) are in boldface and italics. Putative −10 sequences are underlined. The PDR (pentanucleotide direct repeats) upstream of −10 region are in boldface and separated by 6 nucleotides.

3,654.7 Da (Fig. 2). The peaks with *m/z* values of 3,654.7, 3,715.8, 3,777.9, 3,839.9, and 3,902.0 Da resulted from addition of 3, 4, 5, 6, and 7 molecules of ethanethiol to unmodified salivaricin D ($M+H^+ = 3,467.5$ Da) (Fig. 2), respectively. The remaining peaks were consistent with sodium or potassium adducts of the above set of peaks.

Bioinformatics analysis of the salivaricin D locus. The genome of *S. salivarius* 5M6c was sequenced in order to identify the genes responsible for salivaricin D production. Homology searches identified putative LanA, LanB, and LanC family protein-encoding genes carried by the contig 00059 of the draft genome sequence of the strain. Further analysis of the contig 00059 revealed a complete lantibiotic biosynthesis locus, including genes responsible for modification, immunity, export, and regulation of salivaricin D (Fig. 3A).

The salivaricin D locus consists of 12 bacteriocin-related putative open reading frames (ORFs) and two transposase-related genes (Fig. 3A). Each ORF is preceded by a strong ribosome binding site (RBS), and all genes (except *orf1* and *orf2*, which are transposase-related genes) encode proteins required for the production of salivaricin D. The 12 ORFs related to bacteriocin production include genes that encode the putative salivaricin D precursor (*slvD*), a nisin family-like precursor bacteriocin (*slvN*), a dehydratase (*slvB*), an ABC transporter (*slvT*), a cyclase (*slvC*), a protease (*slvP*), a response regulator (*slvR*), a sensor histidine kinase (*slvK*), and four immunity proteins (*slvI*, *slvG*, *slvE*, and *slvF*). All of the ORFs (with the exception of *slvG*, *slvE*, *slvF*, and *orf2*) were encoded on the same DNA strand. The ORFs appear to be organized into 4 operon-like structures (Fig. 3A), all of which contain a putative −10 box and, instead of a −35 box, a pair of pentanucleotide direct repeats (PDR), or a nis-box separated by 11 nucleotides, was identified upstream of the −10 box in the promoter regions. A putative −10 box accompanied by one PDR was also identified upstream of *slvR*, but these were located within the coding region of *slvP*. The PDR are potential binding sites for the response regulator (Fig. 3B) (24).

The first operon consists of the structural gene for salivaricin D (*slvD*). The second operon contains a structural gene for salivaricin N (*slvN*), which is located 148 nucleotides downstream of *slvD*. The third operon (*slvBTCIPRK*) includes genes that encode proteins required for modification of salivaricin D (*slvB* and *slvC*) and salivaricin D export (*slvT*), a protease (*slvP*), an immunity protein (*slvI*), and regulatory proteins (*slvR* and *slvK*). The fourth operon, which is located on the other DNA strand, consists of genes encoding additional immunity proteins (*slvF*, *slvE*, and *slvG*).

There are two regions in the locus, each of which is 117 nucleotides long, that share 86% nucleotide identity with each other. The first starts 20 bp upstream of the start codon of *slvD* and ends 94 bp downstream of the start codon, and the second starts 20 bp upstream of the start codon of *slvN* and ends 94 bp downstream of its start codon. Thus, 97 bp of each of the two regions lies in the coding regions of *slvD* and *slvN*, and they share 87% identity at the nucleotide level. Comparison of the deduced peptide (the first 32 amino acids encoded by *slvD* and *slvN* each) from these coding regions revealed 81% identity. Thus, the salivaricin D structural gene (*slvD*) and salivaricin N (*slvN*) appear to be partial duplications.

The *slvD* gene consists of 171 nucleotides that encode the 57-amino-acid precursor of salivaricin D, which consists of a 23-amino-acid leader peptide and a 34-amino-acid mature peptide (Fig. 4). The leader peptide contains the conserved motif Phe-Asn-Leu-Asp, which is a characteristic feature of class Ia subclass I lantibiotics (9). It differs from the leader of nisin Q by 2 amino acids and from those of nisin A, Z, or F by 5 amino acid substitutions (Fig. 4). The mature peptide has significant similarity to the nisin type of lantibiotics, with 62% identity to nisin Q (57) and nisin Z (31), 59% to nisin A (6) and nisin F (11), and 55% identity to nisin U (56). The identities lie mainly in the N terminus, while differences were found close to the C terminus (Fig. 4). The mature peptide of salivaricin D contains seven hydroxy amino acids (Fig. 4), which matches with the number of dehydrated residues determined by ethanethiol derivatization, indicating that salivaricin D is encoded by *slvD*; identified in the contig. Also, the deduced structure suggests that salivaricin D contains 4 thioether bridges, which are composed of two Lan (Ala-S-Ala) and two MeLan (Abu-S-Ala) amino acids as well as two Dha amino acids and one Dhb (proposed structure shown in Fig. 5).

BlastX analysis identified protein homolog matches for the other ORFs encoded by the salivaricin D gene locus (Table 3), most of which are similar to the gene products of the nisin Q locus.

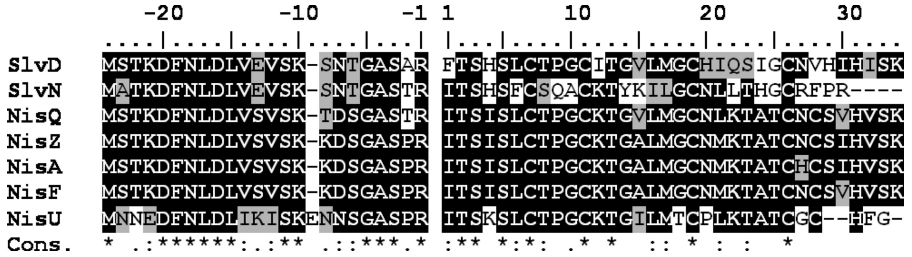

FIG 4 Alignment of salivaricins D and N with similar lantibiotics. The left and right blocks of the alignment represents the leader and mature peptides, respectively.

The *slvN* translation product appears to be a lantibiotic-like peptide (salivaricin N) and shows significant similarity (65% identity and 80% similarity) to nisin Q and salivaricin D precursors (Fig. 4). The mature peptide is 52% and 41% identical to those of nisin Q and salivaricin D, respectively. However, the leader sequence of salivaricin N shows greater identity to salivaricin D (91%) than to nisin Q (78%).

Heterologous expression of salivaricin D immunity gene. In order to confirm that the *slvI* gene encodes the immunity protein of salivaricin D, *slvI* was cloned in the *L. sakei* NCDO 2714 indicator strain on the pMG36e vector. The resulting clone (pLG500) was 32-fold more resistant to purified salivaricin D than *L. sakei* NCDO 2714 carrying only the cloning vector (data not shown).

DISCUSSION

S. salivarius produces many bacteriocins, mainly lantibiotics, most of which are called salivaricins (22, 33, 39, 52–54). Salivaricins have similar primary structures that consist of 22 to 25 amino acids, and their masses range from 2,315 to 2,767 Da (52). In this study, we report the purification and characterization of a new lantibiotic bacteriocin (salivaricin D), which is different from other bacteriocins of *S. salivarius* in both size and sequence. Because of the difference in the primary structures between salivaricin D and other salivaricins, they belong to different subclasses of lantibiotics (subclass I and subclass II). However, salivaricin D and many other salivaricins are similar in that they inhibit *S. pyogenes* and *S. pneumoniae* (52). Thus, salivaricin D and other salivaricins may have potential applications in the control of infections caused by *S. pyogenes* and *S. pneumoniae* (14, 48).

Even though salivaricin D shares significant similarity with the nisins, it may not be regarded as a natural variant of nisins because (i) the variation in amino acid sequence is so large and (ii) salivaricin D possibly lacks ring E (Fig. 5). By definition, natural variants must have only a few amino acid substitutions and the same ring pattern, and producer cells must be immune to the variants (42). Salivaricin D is thus a novel bacteriocin.

Because of its similarity in its presequence to the pronisins, especially to those residues involved in thioether linkage formation (Fig. 4), we predicted the structure of salivaricin D (Fig. 5) using the structure of nisin A as a model. Nisins contain 5 cysteines at positions 7, 11, 19, 26, and 28, while salivaricin D has 4 cysteines, whose positions are conserved, but lacks the cysteine at position 28, which is replaced by a valine residue. Nisins A, Z, Q, and F have 5 Thr residues at positions 2, 8, 13, 23, and 25 and 4 Ser residues at positions 3, 5, 29, and 33; in contrast, in salivaricin D, residues at positions 25 and 29 are replaced by Gly and His, respectively (Fig. 4). This means that the four ring structures [residues 3 and 7 (A), 8 and 11 (B), 13 and 19 (C), 23 and 26 (D)] in nisin A are completely conserved and that only the last MeLan structure (ring E, which involves residues 25 and 28) in salivaricin D is missing. Thus, salivaricin D apparently may form 4 rings (A to D), in contrast to the nisins, which form 5 rings (A to E), and this has to be verified experimentally. However, salivaricin D and nisins may have differences in killing activity, since a single amino acid substitution can change/abolish the activity of a protein. Therefore, the inability of salivaricin D to inhibit listeria, staphylococci, and enterococci may be associated with the lack of this 5th C-terminal ring (ring E) or other differences in amino acid residues of the C-terminal region.

The genetic makeup of the salivaricin D locus is similar to that of class I lantibiotics, especially the nisins. The salivaricin D locus contains all of the genes present in the nisin locus but in a different arrangement. In nisin A, Z, and Q loci, the genes occur in the order *nisABTCIPRKFEG*, while in nisin U, the genes occur in the order *nisPRKFEGABTCI*. In contrast, the salivaricin D locus is separated into two gene clusters found on different DNA strands, such that the *slvANBTCIPRK* cluster is located on one strand while the *slvFEG* cluster is located on the opposite strand. The two clusters are separated by a transposase-like encoding gene (*orf2*), suggesting that the locus was possibly inserted in the genome by a transposon. Trans-

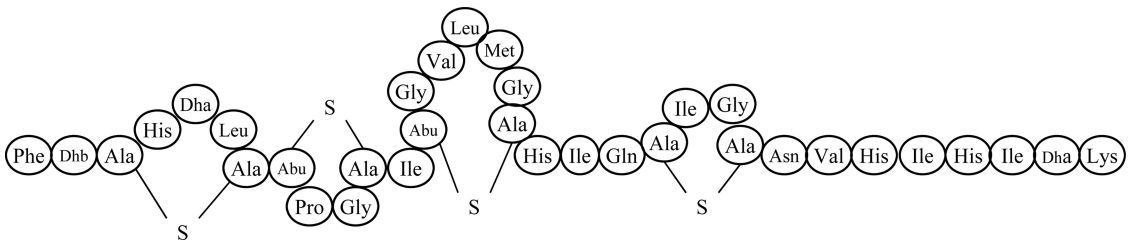

FIG 5 Predicted structure of salivaricin D. Abu, aminobutyric acid; Dhb, didehydrobutyrine; Dha, didehydroalanine.

TABLE 3 Proteins showing similarity to ORF products of salivaricin D locus[a]

ORF	Length (no. of aa)	Similar protein data							
		Designation	Length (no. of aa)	Producer	% identity	% similarity	E value	Function	Reference/GenBank accession no.
orf1	69	COG2963	172	*Streptococcus mitis* NCTC 12261	65	80	1×10^{-5}	Transposase	ZP_07639005
slvD	57	NisQ	57	*Lactococcus lactis* 61-14	62	79	3×10^{-15}	Nisin Q precursor	57
slvN	53	NisQ	57	*L. lactis* 61-14	66	80	1×10^{-9}	Nisin Q precursor	57
slvB	984	NiqB	993	*L. lactis* 61-14	52	73	0	Lantibiotic dehydratase	57
slvT	612	NiqT	600	*L. lactis* 61-14	62	83	0	ABC transporter	57
slvC	406	NiqC	418	*L. lactis* 6F3	53	75	3×10^{-120}	Cyclase enzyme	16
slvI	244	NiqI	245	*L. lactis* 61-14	37	61	2×10^{-35}	Immunity protein	57
slvP	538	NiqP	695	*L. lactis* 61-14	66	83	7×10^{-137}	Leader peptidase	57
slvR	228	NiqR	228	*L. lactis* 61-14	68	83	9×10^{-86}	Response regulator	57
slvK	445	NiqK	447	*L. lactis* subsp. *lactis* KF-14	53	71	6×10^{-122}	Sensor histidine kinase	45
orf2	294	DUF772	449	*Lachnospiraceae bacterium*[b]	50	69	1×10^{-69}	Transposase	ZP_07955024
slvG	216	NisG	214	*S. salivarius* CCHSS3	72	86	4×10^{-107}	Not given	57
slvE	247	NisE	247	*S. salivarius* CCHSS3	90	94	3×10^{-147}	Not given	ZP_08069789
slvF	224	NisF	225	*Streptococcus vestibularis* ATCC 49124	92	96	3×10^{-113}	ABC transporter	ZP_08069789

[a] aa, amino acids.

posase genes have also been associated with the loci of nisins U, A, and Z (16, 27, 56).

Many lantibiotics, including nisin A, nisin U, subtilin, and salivaricin A and its variants, have been shown to autoregulate their own production through a two-component regulatory system involving a response regulator and a histidine protein kinase (24, 26, 43, 49, 53, 56). Nisin has been shown to induce not only its own production but also the production of its associated immunity proteins (17). The presence of genes encoding these two regulatory proteins, as well as a nis-box in the salivaricin D locus, suggests that salivaricin D might act as its own signal peptide, autoregulating its own production and immunity. However, this presumption must be proved experimentally. Interestingly, the nis-box (TCTGA) does show some similarity (indicated by boldface) to direct repeats identified in promoters of the avicin A locus (ATT**TC**A**TGA**), which are also putative binding sites for response regulators (4), suggesting that response regulators for class I and class II bacteriocins could bind to similar DNA sites and might be involved in cross-functions.

The finding that heterologous expression of the immunity gene (*slvI*) in a sensitive indicator strain resulted in reduced sensitivity to salivaricin D indicates that *slvI* confers immunity function. Full immunity to lantibiotics is usually provided by two systems: the lipoprotein LanI(H) and the ABC transporter LanFE(G) (15).

One difference between salivaricin D and the nisin family lantibiotics is that salivaricin D is naturally trypsin resistant. This resistance is advantageous if the producer of salivaricin D is to be used as a probiotic, since it cannot be destroyed by intestinal trypsin and may still exert its antimicrobial activity against ingested pathogens. Trypsin-resistant bacteriocins can also be produced by bioengineering of natural ones, although this might result in reduced activity (34). Engineering of nisins has generated variants with improved activity toward selected pathogens and has identified the hinge region as vital for specificity toward target bacteria (19). Salivaricin D contains His20, Ile21, and Gln22 in the hinge region. The engineered nisin variants with M21I showed enhanced activity against *S. aureus* strains, while N20H and K22Q showed reduced activity compared to natural nisin A (18), and salivaricin D does not inhibit *S. aureus*.

The second lantibiotic gene (*slvN*) found next (similar) to the *slvD* gene appears to be a partial duplication of *slvD*. Duplications of the structural genes for the lantibiotics mutacin III and salivaricin G32 have been identified (36, 52), and none of these have been reported to possess bacteriocin activity. The findings suggest that the duplicate genes identified may have other unknown functions (36). One possible function is that the *slvN* gene may be reminiscent of a two-peptide bacteriocin, which has become a one-peptide bacteriocin. Presently, we do not have any evidence for an active two-peptide bacteriocin. One may also speculate that *slvN* may encode the induction peptide that activates the *slv* locus.

Although *S. salivarius* is predominantly found in the human oral cavity (23, 40), it has also been occasionally isolated from the gut (25, 33, 35, 51), and salivaricin D is produced by a strain which was isolated from the feces of a healthy human infant, indicating that it forms part of the normal flora in the gut as well. In addition, the producer strain appears to occur in high numbers in fecal microflora, suggesting that it might have traits that make it competitive and dominant in the gut flora. For these reasons, *S. salivarius* 5M6c might be a potential probiotic bacterium, and further investigations should be done to evaluate its potential as a probiotic. It should also be pointed out that oral/gut streptococci appear to be a good source for bacteriocins, and this may be used to protect the host against invasion of pathogenic streptococci.

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